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ORIGINAL ARTICLE

Preparation, characterization and *in vitro* evaluation of sterically stabilized liposome containing a naphthalenediimide derivative as anticancer agent

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Abstract

The aim of this study was to incorporate a new naphthalenediimide derivative (AN169) with a promising anticancer activity into pegylated liposomes to an extent that allows its *in vitro* and *in vivo* testing without use of toxic solvent. AN169-loaded liposomes were prepared using the thin-film hydration method and characterized for size, polydispersity index, drug content and drug release. We examined their lyophilization ability in the presence of cryoprotectants (trehalose, sucrose and lysine) and the long-term stability of the lyophilized products stored at 4 °C for 3 and 6 months by particle size changes and drug leakage. AN169 was successfully loaded into liposomes with an entrapment efficiency of 87.3 ± 2.5%. The hydrodynamic diameter of these liposomes after sonication was ~145 nm with a high degree of monodispersity. Trehalose was found to be superior to the other lyoprotectants. In particular, trehalose 1:10 lipid:cryoprotectant molar ratio may provide stable lyophilized liposomes with the conservation of physicochemical properties upon freeze-drying and long-term storage conditions. We also assessed their *in vitro* antitumor activity in human cancer cell lines (HTLA-230 neuroblastoma, Mel 3.0 melanoma, OVCAR-3 ovarian carcinoma and SV620 prostate cancer cells). However, only after 72 h incubation, loaded liposomes showed almost the same IC_{50} as free AN169. In conclusion, we developed a stable lyophilized liposomal formulation for intravenous administration of AN169 as anticancer drug, with the advantage of avoiding the use of potentially toxic solubilizing agents for future *in vivo* experiments.

Keywords

Cancer therapy, DNA-intercalating ligands, drug delivery system, intravenous administration, naphthalenediimide

History

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Introduction

Liposomes are extensively used as drug carriers since their first description by Alec Bangham and colleagues in 1965. The application of liposomes as drug delivery system has found significantly clinical applications due to their versatility and biocompatibility. Moreover, they display the ability to encapsulate drugs in their aqueous interior or to bind pharmaceuticals into or onto their lipid bilayer (Torchilin, 2005). However, from the appearance of early papers, many efforts have been done to overcome a number of problems associated with the *in vivo* use of the conventional liposomes. A great disadvantage of first formulation was the rapid clearance from circulation by uptake into cells of the mononuclear phagocyte system. More recently, significant increase in circulation time was obtained by grafting of PEG to the liposome surface (Immordino et al., 2006). The increase of surface hydrophilicity imparted by PEG avoids opsonisation and allows vehicle distribution to other tissue with a preferential accumulation in solid tumours through the ''enhanced permeability and retention'' effect (Milla et al., 2012). These long-circulating liposomes were termed ''Stealth'' liposomes.

Today, there are many marketed liposomal formulations for the treatment of cancer patients, for example, liposomal doxorubicin Caelyx® (Schering-Plough Corporation, Kenilworth, NJ), Doxil® (Janssen Biotech, Inc., Horsham, PA) and vincristine Marqibo® (Talon, San Francisco, CA), the most recent liposomal formulation to receive FDA approval (Allen, 2013). *In vivo* administration of such PEG-coated phospholipid vesicles represents a useful and less toxic tool to increase the therapeutic potential of anticancer drugs.

At the same time, the search for new compounds with antitumor activity has become one of the main goals in medicinal chemistry. One group of antiproliferative agents used in cancer therapy comprises molecules that intercalate DNA such as naphthalimides. Among these, the earliest drug amonafide entered in clinical development, failed to reach Phase III because of its toxicity. The therapeutic properties of naphthalimides were subsequently improved by designing bis-intercalating agents like naphthalenediimides (Ingrassia et al., 2009).

Recently, we have synthesized a new series of naphthalenediimide derivatives endowed with two basic side chains with the aim to enhance the interactions with the phosphate

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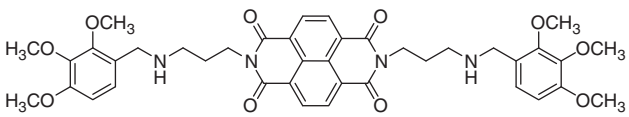

Figure 1. The chemical structure of AN169.

groups of DNA (Tumiatti et al., 2009). Moreover, we tried to combine structural components of several antitumor compounds into a single molecule valuating the influence of different substituents on the aromatic rings towards different cancer cell lines. Among the different molecules, submitted to the Developmental Therapeutics Program at National Cancer Institute for evaluation, one showed an interesting anticancer activity against 60 human cancer cell lines. This compound, named AN169 and characterized by a side chain length of three methylene units and by two 2,3,4-trimethoxy benzyl groups (Figure 1), was further investigated to elucidate the molecular pathways involved in its growth inhibiting activity. The drug was not patented to let its use to the all the scientific community for better elucidate its mechanism of action (Milelli et al., 2012).

However, one of the most serious limitations regarding the pharmaceutical use of this new anticancer drug is was its low solubility in physiological liquids. In particular, the *in vitro* and *in vivo* assays needed dimethyl sulfoxide as solubilising agent.

Therefore, in an attempt to avoid the use of organic solvent, that does not allow a proper assessment of drug activity and cytotoxicity, we propose here a liposomal system as a carrier for AN169. More precisely, in this study, we report on the preparation and characterization of AN169-loaded liposomes.

The objective of this study was to develop lyophilized AN169-loaded liposomes that should have certain desirable characteristics, such as intact cake without volume shrinkage, short reconstitution time (instantaneous or upon gentle manual shaking), desired size and polydispersity, acceptable residual moisture level and preserved drug loading upon lyophilization process and during storage. We also assessed the effect of the optimized liposome formulation on the growth of several human cancer cell lines *in vitro*. The relevance of our novel liposomal nanomedicine could be in providing a new tool for adjuvant therapy of cancer.

Methods

Materials

All the chemicals were purchased from Sigma Chemical Co. (St. Louis, MO,). Hydrogenated soy phosphatidylcholine (HSPC), cholesterol (CHOL), 1,2 distearoylglycero-3-phosphatidylethanolamine-N-polyethylene glycol-2000 (DSPE-PEG) were from Avanti Polar Lipids, Inc. (Alabaster, AL). [^{3}H]cholesteryl hexadecyl ether (^{3}[H]CHE) was obtained from Perkin-Elmer Italia S.p.A. (Monza, Italy). AN169 was synthesized as reported previously (Milelli et al., 2012).

Liposome preparation

Initially, multilamellar vesicles (MLVs) were prepared according to the film hydration method using a standard initial molar ratio of lipids (Pagnan et al., 1999; Di Paolo et al., 2013). Briefly, HSPC:CHOL:DSPE-PEG, 2:1:0,1 molar ratio, were dissolved in chloroform at 10 mM. In some preparations, ^{3}[H]CHE was added as a non-exchangeable, non-metabolizable lipid tracer to assess the lipid content of liposomal dispersion using a scintillation counter of beta luminescence (RadTech, Italy). Lipid was quantified by liquid scintillation counting (LSC) of samples containing a known quantity of ^{3}[H]-CHE. AN169 was dissolved in methanol and then combined with lipids at various drug concentrations (0.25, 0.50, 1.0, 2.0 mg/mL). The ratio of methanol and chloroform was 1:3. The lipid–drug mixture was deposited as a thin film in a round-bottom flask by roto-evaporating the organic solvents under vacuum. The vacuum was applied for 1 h to ensure total removal of trace solvent. The film was hydrated at a temperature above the gel–liquid transition temperature of the amphiphiles (Tc) with 2 mL of phosphate-buffered saline (PBS) solution. The resultant MLVs suspension was further sonicated (Sonopuls HD 2070, Bandelin electronic GmbH & Co. KG, Berlin) using a titanium probe (exponential micro-probe end diameter 3 mm) slightly immersed into the preparation. All the samples were sonicated for 8 min at a power rating of 6 units at a temperature above the Tc. This transformed the milky suspension to a translucent suspension of small unilamellar vesicles (SUVs). After sonication, the samples were left to stand at 20–25 °C (above the Tc) for 30 min prior to proceed with subsequent experiments.

Determination of drug loading in liposomes

Each vesicle suspension was purified from non-incorporated AN169 by gel chromatography on Sephadex G50 pre-equilibrated with Hepes buffer (25 mm HEPES, 140 mm NaCl, pH = 7.4). As gel chromatography isolation process is based on molecular weight, liposomes pass more freely, due to size restriction. On the contrary, free drug may enter the pores in the bead, and the result is that a larger fraction of the overall volume of the column is available to the drug, which thus spends a longer time on the column and is eluted by the mobile solvent after liposomes. After elution of the column void volume, we obtained five opalescent fractions that collected together had a volume of about 2.50 mL. To assess the entrapment efficiency (EE%), expressed as a percentage of the total amount of AN169 found in the studied formulations at the end of the preparation procedure, liposomes were at first disrupted, and then drug content was determined by assaying for incorporated drug by Heλios Thermo spectronic UV–Visible spectrophotometer. Briefly, small aliquots of liposomes (from 300 to 600 nmol of phospholipids) were diluted to 2 mL with methanol, subjected to a bath type sonication until liposomes disruption (10 min) and analysed for drug content at $\lambda = 377$ nm. For the preparation of the calibrations curves, each concentration was analysed five times ($n = 5$). The EE% was calculated from the amount of incorporated drug divided by the total amount used at the beginning of preparation multiplied by 100.

Dynamic light scattering (DLS) measurements

The Z-average particle size (hydrodynamic diameter), size distribution and polydispersity index (PI) were determined by

dynamic light scattering (DLS) spectrophotometric measurement with a 90Plus Nanoparticle Size Analyzer (Brookhaven Instruments, Holtsville, NY) equipped with a 50 mW He-Ne laser (532 nm) at 25 °C. The scattering angle was fixed at 90°. Results were the combination of three 10-minute runs for a total accumulation correlation function (ACF) time of 30 minutes. Prior to analysis, about 0.1 mL of fresh or rehydrated liposomal suspension sample was diluted with 3 mL of Hepes buffer immediately after preparation. Changes in mean diameter were also monitored during leakage experiments carried out at 4 °C for 14 days to assess the stability of liposome formulations in storage conditions. Withdrawals of small aliquots (20 μL) of liposome suspensions were diluted with Hepes buffer and analysed at days 1, 2, 3, 7, 10 and 14. Each measurement was repeated three times.

Freeze drying of liposomes

Freshly prepared SUVs with or without the addition of AN169 were mixed with different cryoprotectants: trehalose, lysine, sucrose at 1:6, 1:8, 1:10, 1:12 lipid:cryoprotectant (L:C) molar ratios. Briefly, 0.1 mL of liposome suspension were mixed with aliquots amounts of the cryoprotectant stock solutions to achieve a final lipid concentration of 2.5 mm and quickly frozen with iced acetone, stored in a ultra low freezer at –80 °C overnight and lyophilized for 48 h using an ALPHA 1-4 LDplus freeze dryer (Christ Osterode, Germany) with a condenser temperature of –55 °C. The dried samples were stored at 4 °C for extended time period under nitrogen atmosphere and reconstituted with sterile water to their original concentrations prior to use (Kundu et al., 2012). Suspensions were observed and evaluated for the rate of reconstitution. For those samples, which failed to rehydrate instantaneously upon addition of water by manual shaking, reconstitution was attempted by vortexing for 1 min. The resulting solutions were characterized and the results were compared to the characteristics of the fresh formulations.

Moisture content analysis using thermogravimetric technique

Thermogravimetric studies were carried out to measure the moisture content of the loaded freeze-dried liposomes using a TGA/SDTA 851e (Mettler Toledo Instruments) equipped with STARe software. The thermogravimetric profiles were recorded with a heating rate of 20 °C/min from 25 to 140 °C under a nitrogen atmosphere (50 mL/min). The sample weights ranged from 4 to 10 mg. The values reported are the percentage loss in weight of the product.

Leakage and in vitro drug release stability of liposome formulations

Leakage of AN169 from reconstituted samples were studied at 4 °C in HEPES buffer pH 7.4 directly after lyophilization and after 3 and 6 months of storage at 4 °C by dialysis bag diffusion technique for 14 days, and compared to freshly prepared formulations. One millilitre of the loaded suspensions was placed in the dialysis bag (Spectra/Por Float-A-Lyser G2, CE, m.w. cutoff: 100 kDa) hermetically sealed and immersed into 20 mL of HEPES buffer pH 7.4. Samples were withdrawn from the receptor compartment at predetermined time intervals and replaced with fresh medium. Aliquots were diluted with acetonitrile before being transferred to HPLC for the determination of drug content. The HPLC assays were carried out by a Nova-Pak C18 (150 x 3.9 mm, 4 mm, Waters) column, UV detector at 377 nm. The mobile phase was a mixture of water (20%) and acetonitrile (80%); the injecting volume 20 μL; the flow rate 1.0 mL/min. The system was thermostated at 37 °C. In these conditions retention time of AN169 was 9 min.

Release studies were carried out in HEPES buffer and 25% FBS (Foetal Bovine Serum) at 37 °C for 72 h on both freshly prepared formulations and on reconstituted liposomes from lyophilized powders at selected time points (directly after freeze-drying, and after 3 and 6 months of storage at 4 °C) by a dialysis bag as above. The entire system was kept in thermal bath with constant stirring. The cumulative release and the drug leakage percentage were calculated by dividing the cumulative amount of drug recovered in the dialysis medium with the total amount in the liposomes. The samples were protected from the ambient light during both leakage experiments and drug release studies.

In vitro cytotoxicity assay

HTLA-230 neuroblastoma, Mel 3.0 melanoma, OVCAR-3 ovarian carcinoma and SV620 prostate cancer cells (donated by Dr. L. Raffaghello, G. Gaslini Institute, Genoa, Italy) were grown in Dulbecco's modified Eagle medium (Euroclone, Milan, Italy) supplemented with 10% foetal bovine serum (GIBCO, Milan, Italy) and 50 UI/ml penicillin, 50 μg/ml streptomycin and 2 mm L-glutamine (Euroclone, Milan, Italy). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air.

The cytotoxicity of AN169-loaded liposome was compared to that of free AN169. Briefly, drug stock solutions (1×10^{-2} M) were prepared in cell culture DMSO. These were initially diluted in phosphate buffer saline (1×10^{-3} M) and finally in complete growth medium (1×10^{-4} M). Several μL were taken and put into cell culture plates to obtain different drug concentrations (highest final DMSO concentration was 0.05%). Liposomes were only diluted in growth medium. All controls contained the highest DMSO concentration used in the treated samples.

Cells were plated in 96-well tissue culture plates, allowed to attach 24 hours, and then left untreated or treated with growth medium containing 0.25, 0.50, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0 μM of free or encapsulated AN169. Briefly, 15 μL of 5 mg/mL 3 -(4,5-diphenyltetrazolium) bromide (MTT) in PBS were then added to each well and cells were incubated for 4 h at 37 °C. Formazan crystals were made soluble with DMSO. Optical densities were determined at 570 nm using a Dynatech MR5000 plate reader. Viability was expressed as a percentage of control by dividing the absorbance of each treated well by the average of the untreated controls. Cytotoxicity was determined by plotting cell viability versus drug concentrations. Results, derived from three different experiments, are expressed as mean percentage from quadruplicate wells as compared to that of control cells. The IC_{50}

of the tested compounds were estimated graphically from the dose-response curves after 24 and 72 h of drug exposure.

Statistical analysis

Data were subjected to statistical analysis using the one-way analysis of variance, and p values <0.05 were regarded as significant. These experiments were expressed as a mean values ± standard deviation (SD).

Results and discussions

Influence of AN 169 concentration on the entrapment percentage and size

AN169 liposomes are intended to be administered by intravenous route (IV). Therefore, their size and loading represent two important parameters to guarantee the extravasation of the therapeutic concentrations of AN169 within the disease site. The thin layer evaporation method was chosen due to its facility to enable the encapsulation of hydrophobic drugs (Deniz et al., 2010). The hydrodynamic diameter of all AN169 liposomes after sonication was ~145 nm with a high degree of monodispersity (Figure 2). The greater amount of AN169 entrapped within liposomes was achieved using an initial AN169 concentration of 2.0 mg/mL. However, the amount of free AN169 to be recovered after the purification step of the production process was high (~0.98 mg/mL). The reduction of the initial concentration of drug to 1.0 or 0.5 mg/mL led to a minor amount AN169 entrapped, but the loss of unentrapped AN169 was lower indicating a better efficiency in the production process. Finally, a further reduction of the initial drug concentration to 0.25 mg/mL led to a low loading of AN169. Thus, the drug concentration used for the preparation of AN169-loaded SUVs was 1.0 mg/mL.

Effects of various cryoprotectants on lyophilized/rehydrated liposomes

The potential application of liposome as therapeutic tool is limited by their inherent chemical instabilities (e.g. lipid oxidation and hydrolysis) and by physical factors such as heating or freezing which accelerate drug release during storage. To circumvent these problems and achieve long-term stability of formulations, freeze-drying (lyophilization) has been used as an effective approach to render liposomes stable without compromising their size or encapsulation capacity (Chengjun et al., 2010).

As first step, loaded and empty liposome formulations were lyophilized without the use of cryoprotectants, as polyethylene glycol has been shown to inhibit nucleation of ice and thus ice crystal formation during freezing (Annunziata et al., 2002). However, PEG-shell alone was not sufficient to protect our stealth liposomes upon lyophilization, thus both size and PI significantly increased (Z-average = 3561 ± 249 nm; PI = 0.86 ± 0.17). Therefore, it was necessary to add cryoprotectants to protect SUVs from fusion during dehydration.

Three potential cryoprotectants were evaluated, sucrose, trehalose and lysine, by determining their effect on liposome size and PI after reconstitution. The freeze-drying efficiency of sugars has been widely confirmed (Crowe et al., 1994), while, more recently, it has been demonstrated that amino acids represent an alternative class, for example, in diabetic patients (Mohammed et al., 2007). As reflected in Table 1, each cryoprotectant used and the molar concentration

(A)

Initial drug concentration (mg/mL)	Mean diameter (nm)	Polydispesity index	Concentration of encapsulated drug (mg/mL)	Entrapment efficiency (%)
2.00	153.8±12.8	0.052±0.055	1.02±0.08[a]	51.4±4.0
1.00	147.4±6.5	0.077±0.015	0.89±0.03[b]	87.3±2.5
0.50	145.2±9.4	0.058±0.03	0.33±0.10[c]	65.9±5.8
0.25	143.9±12.4	0.073±0.01	0.15±0.01[d]	60.5±2.7

(B)

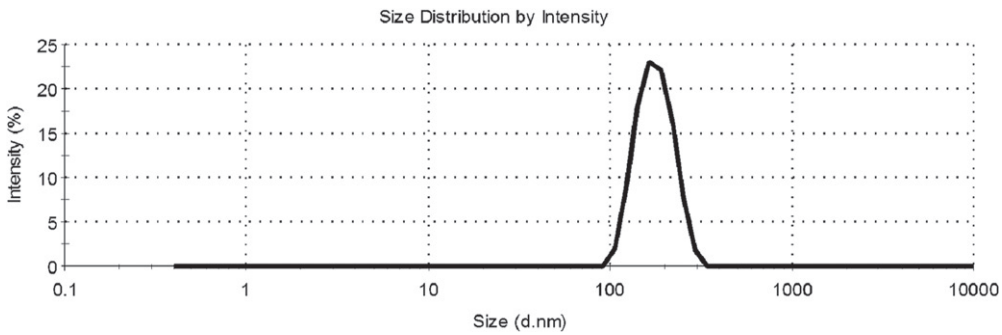

Figure 2. Chemico-physical characterization of freshly prepared SUVs. (A) Influence of different initial AN169 concentrations on liposome size and entrapment efficiency. Values are expressed as the mean ± S.D. ($n = 3$). [a]Significant difference *vs* initial concentration of 2.00 mg/mL ($p > 0.05$). [b]No Significant difference versus initial concentration of 1.00 mg/mL ($p < 0.05$). [c]Significant difference versus initial concentration of 0.50 mg/mL ($p < 0.05$). [d]Significant difference versus initial concentration of 0.25 mg/mL ($p > 0.05$). (B) Representative particle size distribution by intensity of freshly prepared loaded liposomes.

Table 1. Effect of lipid to cryoprotectant (C:L) molar ratio on the vesicle size (nm) and polydispersity index (PI) of loaded SUVs.

	Trehalose			Sucrose			Lysine		
L:C	Size	PI	Reconstitution	Size	PI	Reconstitution	Size	PI	Reconstitution
1:6	280 ± 23	0.357 ± 0.01	Manual shaking	393 ± 34	0.254 ± 0.02	Vortexing	452 ± 49	0.420 ± 0.02	Vortexing
1:8	190 ± 15	0.221 ± 0.05	Instantaneous	220 ± 42	0.179 ± 0.05	Manual shaking	484 ± 18	0.382 ± 0.07	Vortexing
1:10	147 ± 10	0.183 ± 0.03	Instantaneous	153 ± 18	0.198 ± 0.07	Instantaneous	639 ± 57	0.574 ± 0.03	Vortexing

Size and PI were determined by dynamic light scattering. Results shown are the mean ± SD from at least three independent batches of formulation upon reconstitution.

employed influenced cryoprotection efficacy. For example, loaded liposomes, lyophilized in the presence of relatively low 1:6, 1:8 L:C molar concentrations, were smaller compared to unprotected ones, but their mean particle diameters were still significantly increased ($p < 0.05$) compared to liposome size prior to lyophilization; so neither sugars nor lysine were capable of offering protection during freeze-drying. Instead better results were achieved when the two sugars were added at molar ratio of 1:10 L:C with trehalose showing the more efficient stabilizing activity with non-significant difference in size compared to freshly prepared samples ($p = 0.7$ and $p = 0.1$ for trehalose and sucrose, respectively). These findings indicate a successful lyophilization cycle and cryoprotectant, whereas considerable particle growth and broad size distribution were observed with lysine. The amino acid exerted only a slight protection, as liposomes were always significantly larger as compared to freshly prepared ones at each molar ratio tested (p values between 0.01 and 0.03).

Of the evaluated cryoprotectants, for treahlose the results were as expected, because this sugar displays a sufficiently high glass transition temperature ($T'_g = -29\,^\circ C$) and is small enough to interact with phospholipid head groups, thus forming a glassy state during main drying (Hinrichs et al., 2006). Also sucrose, even in a less extend, displays an efficient protection during freeze-drying, while, on the contrary, lysine showed no appreciable effect on the investigated liposome. Lysine has a side chain with a charged positive group, also the drug is characterised by the presence of two amino groups protonated at pH 6–7. It may be possible that the repulsion between the positively charged molecules prevents the protection exerted by lysine on the phospholipid membrane. In fact, comparison of AN169-loaded vesicles with their empty freeze-dried counterparts lyophilized in the presence of lysine revealed a trend of increased vesicle size when AN169 was incorporated within the liposome bilayers. In contrast, the lyophilized empty SUVs and the lyophilized loaded SUVs showed similar behaviour for the two sugars, suggesting that in this case AN169 does not interact with the cushioning effect of cryoprotectants. One reasonable explanation could be that one basic side chain of the AN169 molecule protrudes from the outer margin of the bilayer. This orientation is consistent with the high molecular ratio between the drug and lipids in the final stage of formulation (high drug-entrapment efficiency). Thus, the reason of the different cryoprotection capability among the three compounds may depend on the structural orientation of AN 169 within the lipid bilayer.

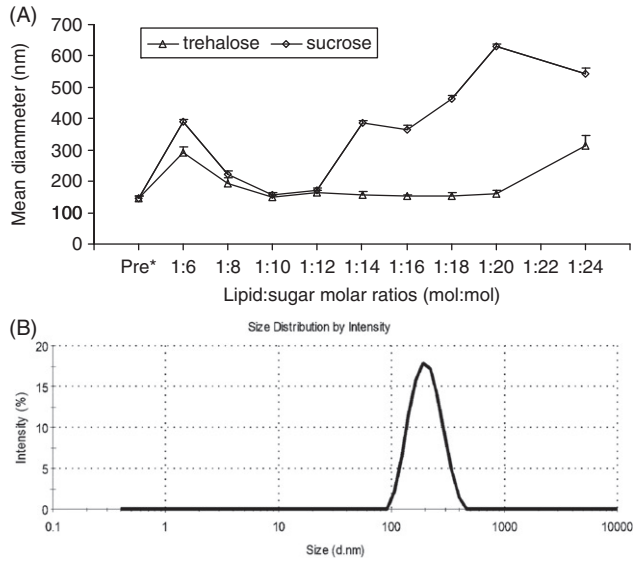

Figure 3. (A) Changes in the mean diameter of reconstituted loaded liposomes as a function of sucrose or trehalose concentrations. *Pre: liposomal size prior to lyophilization. Data are the mean ± SD of three independent experiments. (B) Representative particle size distribution by intensity of lyophilized loaded SUVs cryoprotected with trehalose 1:10 L:C molar ratio.

After selection of cryoprotectant, it is important to optimize the concentration of the used cryoprotectant in such a way that it should be used minimum while preserving all desirable characteristics of the final product. In general, as an increase in sugar to lipid ratio corresponds to a better cryoprotective effect, in an additional experiment we investigated if higher trehalose or sucrose concentrations could improve the cryoprotective capability. As shown in Figure 3, the optimal carbohydrate concentration for stabilizing such liposomes was found to be 1:10 L:C molar ratio. However, further increase in the L:C resulted in a decrease in cryprotection only for sucrose, while, in contrast, loaded SUVs freeze-dried in the presence of trehalose, were still spherical and uniform up to 1:20 L:C molar ratio; vesicles fusion occurred only when molar concentration of 1:24 L:C was employed. This biphasic nature of cryoprotectants has previously been demonstrated (Suzuki et al., 1996), an initial increase in concentration is often associated with a higher uniformity of size while a further increase may often let to aggregation and damage.

In summary, to achieve storage stability of liposomes by freeze-drying procedure the addition of cryoprotectants is absolutely necessary, irrespective of the surface-attached polyethylenglycol, which is reported to be an effective cryo-agent by itself. For lyophilization, good results were obtained

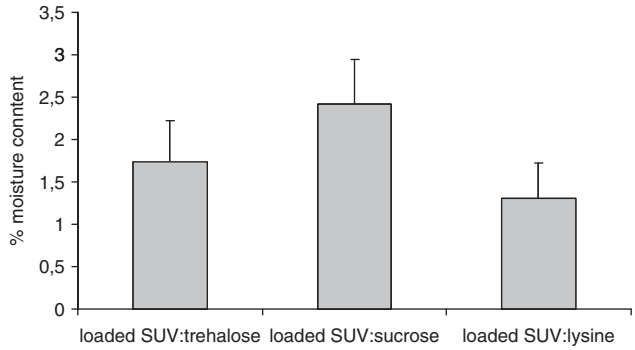

Figure 4. Influence of cryoprotectants on moisture content after freeze-drying. SUVs were freeze-dried with 10 mol of cryoprotectant added per mol of the lipid. Moisture content was assessed by TGA. All the values reported are the mean from three samples ± SD.

with trehalose and sucrose, while lysine seems to be less suitable revealing high polydispersity indexes.

Residual moisture content in the lyophilized loaded liposomes

The physicochemical properties of lyophilized products are influenced by residual moisture content in the dried powders. High values of moisture may also destabilize the delivery systems during storage because of cryoprotectants crystallization (amorphous collapse). Water may induce a shift in the T_g (glass transition temperature) of the formulation to below the temperature of storage, thus high residual water content may initiate crystallization of the formulation during storage and subsequent collapse of the product (Miller, 2006). Acceptable residual moisture values range from 1% to 3% w/w for most pharmaceutical products. Analysis of moisture content of the loaded freeze-dried liposomes revealed that all the formulations tested had low level of residual moisture content immediately post-freeze-drying (Figure 4), in line with the levels globally accepted by the regulatory authorities. Thus, the presence of water concentration below 3% (w/w) in freeze-dried products confers improved stability and drug retention by preventing any chemical reactions that can occur due to hydrolysis.

Drug retention efficiency of lyophilized SUVs

It is important that drug loading of the delivery system should remain stable before and after the process of lyophilization. Moreover, for commercialization of any pharmaceutical formulation, it is a mandatory requirement to have long-term stability of the final product. Hence, further study on the stability not only in connection to the process of lyophilization but also for long-term storage conditions is deemed necessary.

Liposomes, if not efficaciously protected during freeze-drying, can undergo membrane damage due to the formation of ice crystals with extensive drug loss (Kirby, 1984). Furthermore, instability of a liposome system in the drier state, due for example to a gradual increase of residual moisture content during storage, leads to aggregation or fusion of liposomes, which in turn increases particle size and drug leakage. Particles of greater diameter are taken rapidly

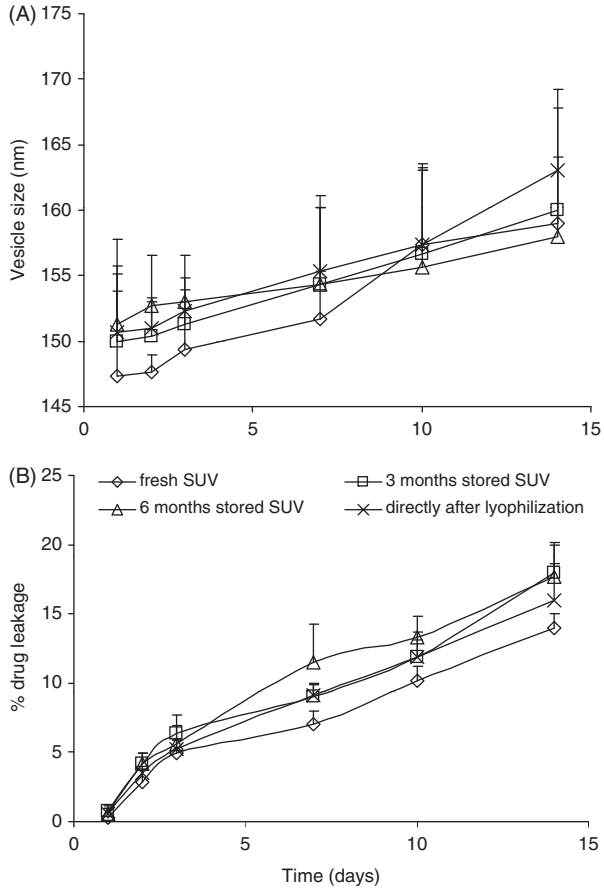

Figure 5. Analysis of leaking from the liposomal carrier at 4 °C. After freeze-drying loaded SUVs cryoprotected with trehalose 1:10 L:C molar ratio were stored in the lyophilized state at 4 °C and at selected time points: immediately after freeze-drying, after 3 months and after 6 months were reconstituted with HEPES buffer. Rehydrated samples were maintained at 4 °C for 14 days and evaluated for vesicle mean diameter (B) and drug loss (B). Release and size profiles were compared to freshly prepared formulations to assess changes in stability after lyophilization and storage conditions. Values are displayed as the mean ± SD ($n = 3$).

by the RES (reticuloendothelial system) resulting in their rapid clearance and short half-life. For this purpose to establish drug leakage under storage conditions we chose the formulation cryoprotected with trehalose 1:10 L:C molar ratio as it was characterized by the best physicochemical properties (size, PI and residual moisture content). After reconstitution in HEPES buffer, the lyophilized powders, stored at 4 °C for 3 and 6 months, were maintained at 4 °C for 14 days and their release profiles were compared to those of freshly prepared suspensions and to those of reconstituted samples immediately after freeze-drying. At the same time, we assessed vesicle size as the retention of entrapped drug could modulate the fluidity and rigidity of the phospholipid membrane.

As shown in Figure 5, drug leakage from fresh suspensions was negligible up to 1 week (7.0 ± 1.7%) with a slight progressive increase. In the case of reconstituted formulations, for any storage time all the samples showed a similar loss of drug suggesting that freeze-drying did not affect damage of the carrier system. In addition, the analysis of the mean size revealed a little gradual aggregation over time demonstrating that the constant maintenance of vesicle diameter is clearly associated with drug retention.

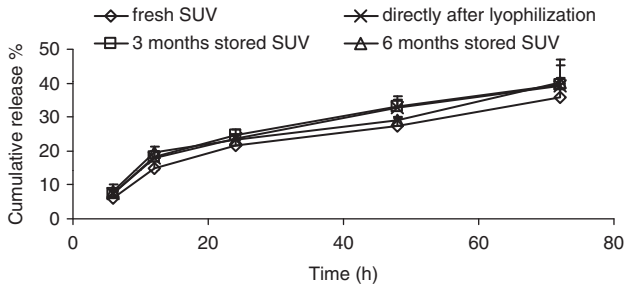

Figure 6. Cumulative AN169 release at 37 °C. After freeze-drying loaded SUVs cryoprotected with trehalose 1:10 L:C molar ratio were stored in the lyophilized state at 4 °C and at selected time points: immediately after freeze-drying, after 3 months and after 6 months were reconstituted with PBS and 25% FBS. Rehydrated samples were maintained at 37 °C for 72 h and evaluated for drug release. Release profiles from reconstituted samples were compared to freshly prepared formulations to assess changes in stability after lyophilization and storage conditions. Values are displayed as the mean ± SD ($n = 3$).

In vitro drug release of SUVs

For any controlled drug release formulation, effectiveness of the preparation itself is primarily based on drug release profile, which should remain in the desired profile for both freshly prepared formulations and after storage. Therefore, in this study, the *in vitro* drug release profiles of freshly prepared liposomes were compared to those that were trehalose-cryoprotected (1:10 L:C) and immediately reconstituted after freeze-drying. At the same time, we studied if any changes in the dried formulation occurred after 3 months and 6 months long-term storage at 4 °C. Figure 6 presents these drug release profiles obtained at 37 °C in PBS and 25% FBS by dialysis method at different storage times. Notably, the differences in the release profiles of lyophilized and non-lyophilized liposomes were no statistically significant, and all types of samples displayed sustained release of AN169, suggesting that neither the process of freeze-drying, nor storage conditions altered the drug release kinetics from PEG-liposomes.

Effect of liposomal AN169 on the growth of cancer cells *in vitro*

The cytotoxic activity of liposomes encapsulated with AN169 was tested through the MTT assay on several cancer cell lines: HTLA-230 neuroblastoma, Mel 3.0 melanoma, OVCAR-3 ovarian carcinoma and SV620 prostate cancer cells. Table 2 summarizes the IC_{50} values of the drug and drug loaded liposomes at two different incubations times. However, the AN169 formulation had higher IC_{50} values (decreased cytotoxicity) compared to unincorporated AN169, while only after 72 h incubation, loaded liposomes showed almost the same IC_{50} as free AN169. These results may be related to the steric hindrance from PEG chains on the liposome surface, and also to the stability of liposome itself that retard the drug release when the vesicles come into contact with medium and cells (Crosasso et al., 2000).

Table 2. Cytotoxic activity of free AN169 and AN169-loaded liposomes against HTLA-230, OVCAR-3, Mel 3.0 and SV620 cells.

		IC_{50}	
Cell line	Exposure time (h)	Free AN169	Encapsulated AN169
HTLA-230	12	0.90 ± 0.04	10 ± 2
OVCAR-3	12	1.67 ± 0.01	20 ± 1
Mel 3.0	12	2.86 ± 0.06	45 ± 6
SV 620	12	1.80 ± 0.01	30 ± 3
HTLA-230	72	0.15 ± 0.08	0.18 ± 0.06
OVCAR-3	72	0.22 ± 0.06	0.29 ± 0.07
Mel 3.0	72	0.75 ± 0.04	0.80 ± 0.01
SV 620	72	0.43 ± 0.03	0.50 ± 0.01

IC_{50} values are the molar concentration causing 50% growth inhibition expressed in μM, evaluated by MTT method. Results, derived from three different experiments in quadruplicate wells as compared to that of control cells, are expressed as mean ± S.E.M.

Conclusions

Among desirable physicochemical characteristics of nanoformulations, size, polydispersity and entrapment efficiency are critical parameters that affect the *in vivo* performance of the drug delivery systems. Our freshly prepared sterically stabilized liposomes are characterized by a size of 147.3 ± 6.5 nm with a polydispersity of 0.077 ± 0.015 and an entrapment efficiency of 87.3 ± 2.5%. Instabilities of liposome suspensions during storage are a serious limiting factor for their applicability as drug delivery system. So, to circumvent this problem, freeze-drying fresh formulations are needed. Here, we have tested three different cryoprotectants, trehalose, sucrose and lysine, but only sugars exerted an efficacious protection during freeze-drying. In particular, the results of this study demonstrated that trehalose 1:10 L:C molar ratio may provide stable lyophilized liposomes with the conservation of physicochemical properties upon lyophilization and long-term storage conditions. This formulation has the advantage of avoiding the use of potentially toxic solvents for future *in vivo* experiments.

Based on these considerations, these results justify further evaluation of AN169-loaded liposomes in cancer treatment.

Declaration of interest

Authors have not declarations of interest to report.

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References

Allen TM, Cullis PR. (2013). Liposomal drug delivery systems: From concept to clinical applications. Adv Drug Del Rev 65:36–48.

Annunziata O, Asherie N, Lomakin A, et al. (2002). Effect of polyethylene glycol on the liquid-liquid phase transition in aqueous protein solutions. Proc Natl Acad Sci USA 99:14165–70.

Chen C, Han D, Cai C, Tang X. (2010). An overview of liposome lyophilization and its future potential. J Contol Release 142: 299–311.

Crosasso P, Cerruti M, Brusa P, et al. (2000). Preparation, characterization and properties of sterically stabilized paclitaxel-containing liposomes. J Control Release 63:19–30.

Crowe JH, Leslie SB, Crowe LM. (1994). Is vitrification sufficient to preserve liposomes during freeze-drying?. Cryobiology 31:355–66.

Deniz A, Sade A, Severcan F, et al. (2010). Celecoxib-loaded liposomes: effect of cholesterol on encapsulation and in vitro release characteristics. Biosci Rep 30:365–73.

Di Paolo D, Pastorino F, Zuccari G, et al. (2013). Enhanced anti-tumor and anti-angiogenic efficacy of a novel liposomal fenretinide on human neuroblastoma. J Control Release 170:445–51.

Hinrichs WL, Mancenido FA, Sanders NN, et al. (2006). The choice of a suitable oligosaccharide to prevent aggregation of PEGylated nanoparticles during freeze thawing and freeze-drying. Int J Pharm 311:237–44.

Kirby CJ, Gragoriadis G. (1984). Preparation of liposomes containing factor VIII for oral treatment of haemophilia. J Microencapsul 1: 33–45.

Kundu AK, Chandra PK, Hazari S, et al. (2012). Stability of lyophilized siRNA nanosome formulations. Int J Pharm 423:525–34.

Immordino ML, Dosio F, Cattel L. (2006). Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. Int J Nanomed 1:297–315.

Ingrassia L, Lefranc F, Kiss R, Mijatovic T. (2009). Naphtalimide and Azonafides as promising anti-cancer agents. Current Med Chem 16: 1192–213.

Milelli A, Tumiatti V, Micco M, et al. (2012). Structure-activity relationships of novel substituted naphthalene diimides as anticancer agents. Eur J Med Chem 57:417–28.

Milla P, Dosio F, Cattel L. (2012). PEGylation of proteins and liposomes: a powerful and flexible strategy to improve the drug delivery. Curr Drug Metab 13:105–19.

Miller DP, Lechuga-Ballesteros D. (2006). Rapid assessment of the structural relaxation behavior of amorphous pharmaceutical solids: effect of residual water on molecular mobility. Pharm Res 23: 2291–305.

Mohammed AR, Coombes AGA, Perrie Y. (2007). Amino acids as cryoprotectans for liposomal delivery system. Eur J Pharm Sci 30: 406–13.

Pagnan G, Montaldo PG, Pastorino F, et al. (1999). GD2-mediated melanoma cell targeting and cytotoxicity of liposome entrapped fenretinide. Int J Cancer 81:268–74.

Suzuki T, Komatsu H, Miyajima K. (1996). Effects of glucose and its oligomers on the stability of freeze dried liposomes. Biochim Biophys Acta 1278:176–82.

Torchilin VP. (2005). Recent advantages with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 4:145–60.

Tumiatti V, Milelli A, Minarini A, et al. (2009). Design, synthesis, and biological evaluation of substituted naphthalene imides and diimides as anticancer agent. J Med Chem 52:7873–7.